PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

To:
NOVO NORDISK A/S
Corporate Patents
Novo Allé
DK-2880 Bagsværd
DANEMARK

Reference	Country
Agent 16. JUL. 1999 McB.	Short title
Action MoBi 20. JUL 1999 EMo 99 07. 19	Term

PCT

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

Date of mailing (day/month/year) 08 July 1999 (08.07.99)	
Applicant's or agent's file reference 5250-WO, MoBi	IMPORTANT NOTICE GMDa 99 07. 16 Notat

International application No. PCT/DK98/00554	International filing date (day/month/year) 16 December 1998 (16.12.98)	Priority date (day/month/year) 23 December 1997 (23.12.97)

Applicant
NOVO NORDISK A/S

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:

   AU,CN,EP,IL,JP,KP,KR

   In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:

   AL,AM,AP,AT,AZ,BA,BB,BG,BR,BY,CA,CH,CU,CZ,DE,DK,EA,EE,ES,FI,GB,GE,GH,GM,HR,HU,ID, IN,IS,KE,KG,KZ,LC,LK,LR,LS,LT,LU,LV,MD,MG,MK,MN,MW,MX,NO,NZ,OA,PL,PT,RO,RU,SD,SE, SG,SI,SK,SL,TJ,TM,TR,TT,UA,UG,UZ,VN,YU,ZW

   The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on 08 July 1999 (08.07.99) under No. WO 99/33964

REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, a **demand for international preliminary examination** must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the **national phase**, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

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Published
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(54) Title: A PROCESS FOR IMMOBILISATION OF ENZYMES

(57) Abstract

A process for producing an immobilized enzyme preparation for use in a mainly organic medium devoid of free water comprising using a fluid bed.

A PROCESS FOR IMMOBILISATION OF ENZYMES

FIELD OF THE INVENTION

The invention relates to a process for producing an immobilized enzyme preparation for use in a mainly organic medium essentially devoid of free water, and use of the immobilized enzyme preparation for organic synthesis.

BACKGROUND OF THE INVENTION

Immobilized enzymes are known to be used for organic synthesis.

The most commonly immobilized enzymes are lipases used for esterification reactions in mainly organic media essentially devoid of free water.

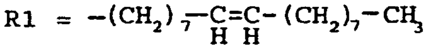
EP 140542 B2 describes a process, wherein an enzyme containing liquid is brought in contact with a weak anion exchange resin carrier by dispersing the carrier in the liquid and mixing by stirring with a magnetic stirrer, whereby the enzyme is immobilized on the carrier. The immobilization is subsequently followed by vacuum drying of the enzyme-carrier.

WO 95/22606 describes a process, wherein an enzyme containing liquid is brought in contact with porous silica carrier by atomizing the liquid onto the carrier in a mixer, subsequently followed by drying overnight a ambient conditions

In industrial immobilization processes described in prior art, the carrier or support material is placed in a column shaped adsorption vessel and an enzyme containing liquid is recirculated until sufficient adsorption of the enzyme on the carrier has been obtained. Following the adsorption step the column is emptied by manually shoveling the enzyme-carrier product into trays. The product is then dried by placing the trays under vacuum at room temperature for a period of 14-16 hours.

WO 94/26883 describes a process for producing dust-free enzyme granules by absorbing the enzyme on a porous material, said material including NaCl, Soda, and silica, and optionally coating the product with a protective outer layer. Generally immobilization of enzymes should not be compared with

Figure 3 shows an example of alcoholysis catalyzed by a lipase, wherein a first reactant (reactant 1), e.g. a triglyceride is reacted with a second reactant (reactant 2), e.g. an alcohol, the substituents R1 and R2 being exchanged in said reaction. R1 and R2 may as an example be:



$$R1 = -(CH_2)_7-\underset{H}{C}=\underset{H}{C}-(CH_2)_7-CH_3$$

$$R2 = -(CH_2)_{16}-CH_3$$

SUMMARY OF THE INVENTION

The present invention provides alternative processes for industrial immobilization of enzymes, which significantly increases capacity and reduces labor costs, by means of standard multi-purpose process equipment.

Thus the invention provides processes for producing an immobilized enzyme preparation for use in a mainly organic medium essentially devoid of free water, which in a first aspect comprises:

a) fluidising a particulate porous carrier in a fluid bed,

b) introducing an enzyme containing liquid medium by atomization into the fluid bed, so as to adsorb the enzyme on the carrier, and

c) removing volatile components of the liquid medium from the carrier in the fluidized bed.

In a second aspect the processes comprise:

a) contacting an enzyme containing liquid medium with a particulate porous carrier with a substantially hydrophobic surface, so as to adsorb the enzyme on the carrier, and

b) introducing a particulate hygroscopic substance, so as to suppress agglomeration of the carrier,

c) removing volatile components of the liquid medium and the hygroscopic substance from said product in a fluidized bed.

Finally in a third aspect the processes comprise:

carriers are described in JP 08126489-A, wherein a water insoluble carrier is coated with a polymer forming a disulphide linkage with enzymes. A third type of carriers is described in *Biotechnology Techniques vol. 3 No 5 345-348,* wherein a ceramic carrier is coated with polyethylene amine, polyethylene imine or 3-aminopropyltriethoxysilane, all three surface types allowing an enzyme to be covalently bound via glutaraldehyd coupling.

In a third embodiment of the invention the carrier particles comprise an organic polymer resin with a substantially hydrophobic surface. The resin may be an adsorbent resin, preferably a polyacrylate, a polymethacrylate (e.g. polymethyl methacrylate), polystyrene cross-linked with divinylbenzene, polyurethane or polypropylene or the resin may be an ion exchange resin, preferably an anion exchange resin, e.g. a weakly basic anion exchange resin. A preferred anion exchange resin is a phenolic type Duolite resin from Rohm & Haas.

Further the carrier may be made from regenerated chitosan as disclosed in DE 4429018-A.

The Enzyme

The enzyme to be immobilized according to the invention may be any enzyme suitable for use in media essentially devoid of free water. The most commonly used enzymes are lipases and in a specific embodiment of the invention the lipase may be derived from a strain of the genus *Humicola* (also known as *Thermomyces*), *Pseudomonas*, *Candida*, or *Rhizomucor*, preferably the species *H. lanuginosa* (also known as *Thermomyces lanuginosa* as described in US 4,810,414 and EP 305216 which are hereby included by reference), *C. antarctica* or *R. miehei*.

Further the lipase may be positionally site specific (i.e. 1,3 specific) or non-specific, upon interaction with triglycerides as substrates.

The enzyme may further be covalently cross-linked by glutaraldehyde treatment during the immobilization process.

The enzyme containing liquid medium

The enzyme containing liquid medium is a hydrophilic medium, preferably aqueous. It may thus contain more than 20%

equipment, e.g. a Uni-Glatt fluidized bed apparatus (Glatt, Germany), thereby removing volatile components.

ii. In a second embodiment of the invention the immobilization of enzyme on a carrier having a substantially hydrophilic surface may alternatively be conducted in a standard fluid bed equipment, e.g. a Uni-Glatt fluidized bed apparatus (Glatt, Germany), wherein the dry porous and particulate carrier is fluidized and an enzyme containing liquid at ambient temperature is introduced by atomization to the fluidized carrier, e.g. using a nebulizer connected to a pump (e.g. a standard peristaltic Watson-Marlow pump). In this embodiment immobilization and drying are conducted simultaneously.

Immobilization on carriers with a hydrophobic surface

Without being bound to the theory it is contemplated that immobilization of enzyme on carriers having a substantially hydrophobic surface involves adsorption of the enzyme on the surface. The immobilization may be enabled by the enzyme forming hydrogen bonds, ionic bonds or covalent bonds with moieties in the surface.

iii. In a third embodiment of the invention the immobilization of enzyme on a carrier having a substantially hydrophobic surface may thus be conducted in a standard mixing equipment, wherein an enzyme containing liquid is introduced to the dry porous and particulate carrier in amount thus forming a paste or a slurry. The paste or slurry is mixed for a period of time in which the enzyme is adsorbed. Following the adsorption step a hygroscopic particulate substance of a particle size smaller than the carrier is introduced to the slurry or paste. Said substance substantially prevents agglomeration of the enzyme-carrier by adsorption of excess liquid, thereby enabling the subsequent drying of the enzyme-carrier product by fluidising said product in a standard fluid bed equipment, e.g. a Uni-Glatt fluidized bed apparatus (Glatt, Suitable temperatures of the inlet air for removing volatile components will primarily depend of the thermal stability of the enzyme (the inactivation temperature). The temperature may be 40-90°C, preferably 50-70°C, e.g. 60°C. A higher temperature provides shorter immobilization and drying times.

Further, time consumption for immobilization and/or drying of the enzyme-carrier when equipment, air inlet flow and air temperature are fixed will depend on the quantity of enzyme-carrier. The immobilization/drying process may be monitored by measuring the air inlet temperature and the air outlet temperature. While the enzyme-carrier is moist the outlet temperature is lower than the inlet temperature due to the heat absorption and evaporation of volatile components. Typically a steady state evaporation occurs during the immobilization/drying process where the outlet temperature stabilizes on a temperature lower than the inlet temperature indicating that evaporation of volatile components (i.e. heat absorption) occurs at a constant rate. At the end of the immobilization/drying process the outlet temperature begins to raise and approaching the inlet temperature indicating that the heat absorption has decreased and thus the moisture of the enzyme-carrier has been removed. Using a fluid bed for immobilization and drying simultaneously the drying process will occur for as long as the enzyme containing liquid is atomized into the fluid bed, and may suitably be extended for 5-30 minutes after inlet of the enzyme containing liquid has ended.

An important aspect of the invention is that the immobilization processes can be easily scaled up by applying other larger standard equipment. Thus the equipment setting ranges given *vide supra* may be adjusted to optimize larger scale equipment.

Uses of immobilized enzyme preparation

Immobilized enzyme prepared in context of the invention may be used for hydrolysis, synthesis or modification of organic substances in a medium essentially devoid of free water. Said

Gum Arabic as emulsifier and tributyrine as substrate) liberates 1 mmole titrable butyric acid per minute.

A folder AF 95/5 describing this analytical method in more detail is available upon request to Novo Nordisk A/S, Denmark, which folder is hereby included by reference.

Trans-esterification assay

a) 200 mg Trilaurin (Fluka) and 571 mg of myristic acid (8 molar equivalents) of myristic acid (Merck) was dissolved in 20 ml heptane. 3 ml saturated NaCl solution was added and the mixture was stirred in a closed bottle for 24 hours at ambient temperature.

b) The immobilized enzyme (50 mg) was water equilibrated in a dissicator (hermetically closed vessel) for 24 hours, using gas phase equilibrium with a saturated NaCl solution (water activity = 0.75).

c) At T = 0 minutes the water equilibrated immobilized enzyme and substrate was mixed in a closed bottle, which was placed in a shaking bath at 40°C. 100 µl samples were withdrawn from the closed bottle using a syringe at T = 0, 10, 20, 30, 40, 50 and 60 minutes. The samples were diluted (1:5 vol:vol) with a 50/50 (% v/v) mixture of acetone/acetonitrile and analyzed on a HPLC system.

Analysis on the HPLC system:

d) The HPLC system was equipped with a LiChrosphere 100 RPC18 endcapped 5 µm (125 x 4 mm) column (Merck). A 50/50 (% v/v) isocratic acetonitrile/acetone solution was selected as the mobile phase with a flow of 1 ml/minute.

e) 20 µl of sample was injected and the formed products (1,2-dilauroyl-3-myristoyl-glycerol (product 1) and 1,3-dimyristoyl-2-lauroyl-glycerol (product 2)) were measured by evaporative light scattering detection (Sedex 55, Sedere, France) at 2 bar pressure and a temperature at 30°C.

f) The amounts of formed products were estimated by comparing sample measurements to external standard curves of 1,2-dilauroyl-3-myristoyl-glycerol and 1,3-dimyristoyl-2-lauroyl-glycerol.

solution was applied via an peristaltic pump (Watson-Marlow)(flow rate 238 g/hour). Inlet air temperature and product temperature were identical with those indicated in example 1. After the immobilization was finished the product was dried for an additional 5 min in the fluid bed.

The immobilization process was tested on the inter-esterification assay, which measured 12% conversion of trilaurin after T = 24 hours

Example 3

Lipase adsorption onto adsorbent resin in mixer and subsequent drying in fluid bed using Hyper Flow Celite (HFC) as drying aid.

94 g of a solution of *Humicola lanuginosa* lipase (693 kLU/ml) was diluted with 260 g demineralized water. The solution was added to 250 g adsorbent resin (a macro-porous divinylbenzene cross-linked polystyrene, Purolite AP 1090; from Purolite, UK) in a 5 l mixer (Lödiger, Germany). The suspension was mixed for 20 minutes at ambient temperature and with RPM of 30. 200 g Hyper Flow Celite (HFC) was added to absorb residual liquid enabling the mixture to be fluidized in the Uni-Glatt apparatus. Using the same conditions as described in example 1, the mixture was dried for 20 min. In this period, the HFC was separated from the adsorbent resin using a 300µm filter on the top of the fluid bed to retain the resin particles while HFC was blown off.

Activity of product:
The rate for T = 0 - 60 minutes was measured to 3,9 U/g product.

Example 4

Lipase adsorption onto adsorbent resin in fluid bed with simultaneous drying of volatile liquids.

94 g of a solution of Humicola lanuginosa lipase (693 kLU/ml) was diluted with 200 g of demineralized water and atomized onto 200 g of adsorbent resin (macro-porous divinylbenzene cross-linked polystyrene, Purolite AP 1090; from Purolite, UK) in a Uni-Glatt (Glatt, Germany) fluidized bed

CLAIMS

1. A process for producing an immobilized enzyme preparation for use in a mainly organic medium devoid of free water, comprising:

a) fluidising a particulate porous carrier in a fluid bed
b) introducing an enzyme containing liquid medium by atomization into the fluid bed, so as to fixate the enzyme on the carrier, and
c) removing volatile components of the liquid medium from the carrier in the fluidized bed.

2. A process for producing an immobilized enzyme preparation for use in a mainly organic medium devoid of free water, comprising:

a) contacting an enzyme containing liquid medium with a particulate porous carrier with a substantially hydrophobic surface, so as to adsorb the enzyme on the carrier, and

b) introducing a hygroscopic substance, so as to suppress agglomeration of the carrier by absorbing excess liquid,

c) removing volatile components of the liquid medium and the hygroscopic substance from said product in a fluidized bed.

3. A process for producing an immobilized enzyme preparation for use in a mainly organic medium devoid of free water, comprising:

a) introducing an enzyme containing liquid medium enzyme by atomization onto a particulate porous carrier with a substantially hydrophilic surface, so as to fixate the enzyme on the carrier, wherein the liquid is introduced in an amount such that substantially no agglomeration of the carrier occurs and

b) removing volatile components of the liquid medium from said product in a fluidized bed.

4. The process of claim 1 or 3 wherein the carrier comprises an inorganic material with a substantially hydrophilic surface, which is essentially insoluble in hydrophilic or hydrophobic liquids or mixtures thereof.

18. The process of claims 1-3, wherein the enzyme is a lipase.

19. The process of claim 18, wherein the lipase is derived from a strain of the genus *Humicola* (also known as *Thermomyces*), *Pseudomonas*, *Candida*, or *Rhizomucor*, preferably the species *H. lanuginosa* (also known as *Thermomyces lanuginosa*), *C. antarctica* or *R. miehei*.

20. A process for enzymatic modification of an organic compound comprising contacting in a reaction medium essentially devoid of free water said organic compound with an immobilized enzyme produced by the process of any of the claims 1-19.

21. The process according to claim 20, wherein the modification is a trans-esterification reaction comprising contacting a first reactant which is a fatty acid ester, a second reactant which is another fatty acid ester, an alcohol or a free fatty acid with an immobilized lipase produced by the process of any of the claims 1-19.

22. The process of claim 21, wherein the first reactant is a triglyceride.

23. The process of claims 21 and 22, wherein the second reactant is a fatty acid ester, and the lipase positionally specific.

24. The process of claim 21, wherein the first and the second reactants are different triglycerides or different mixtures of triglycerides, and the lipase is positionally 1,3-specific.

25. The process of claim 21-24, wherein the reaction medium consists essentially of triglycerides.

26.. The process of claim 21-24, wherein the reaction medium comprises an organic solvent.

Fig. 1: Acidolysis

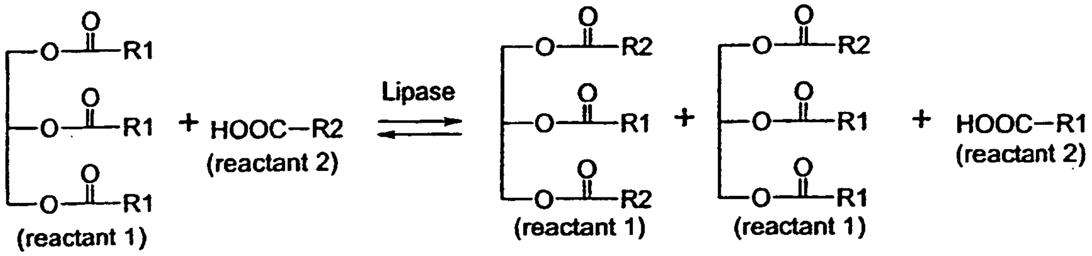


Fig. 2: Inter-esterification

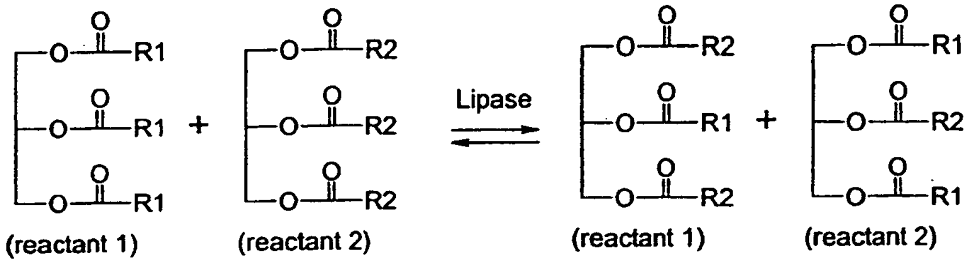
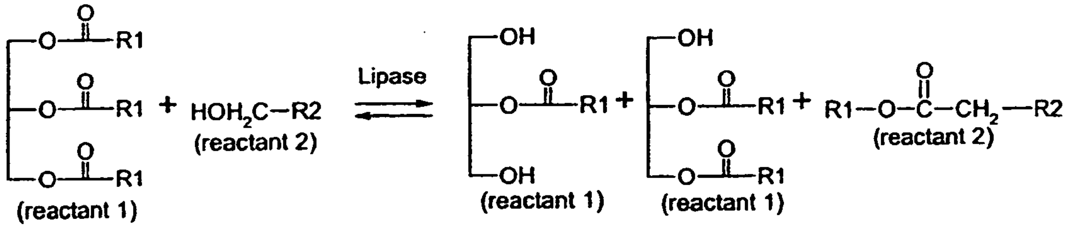


Fig. 3: Alcoholysis



INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 98/00554

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 11/02, C12N 11/14
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9426883 A2 (GIST-BROCADES N.V.), 24 November 1994 (24.11.94), page 5, line 4 - line 8	1,3,6-13, 17-20
A		2,4-5,14-16, 21-26
	--	
A	WO 9522606 A1 (NOVO NORDISK A/S), 24 August 1995 (24.08.95)	1-26
	--	
A	EP 0140542 A1 (NOVO INDUSTRI A/S), 8 May 1985 (08.05.85), example 7	1-26
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☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search 6 April 1999	Date of mailing of the international search report 12 -04- 1999
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Patrick Andersson Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT
Information on patent family members

02/03/99

International application No.
PCT/DK 98/00554

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